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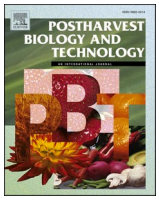

High cold tolerance and differential population response of third instars from the *Zeugodacus tau* complex to phytosanitary cold treatment in navel oranges

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ABSTRACT

The scarcity of phytosanitary treatment options for polyphagous pests of the *Zeugodacus tau* complex poses a quarantine risk for importing countries. Owing to the unresolved taxonomy and a limited number of cold treatment schedules available for this group, we tested whether populations of the *Z. tau* complex infesting *Citrus sinensis* differ in their cold tolerance and determined the efficacy of a single cold treatment against the apparently most tolerant of four populations tested. Additionally, the susceptibility and suitability of *C. sinensis* to *Z. tau* populations were assessed in field cages. Treatment efficacy was determined by larval mortality. Tests comparing the tolerance of *Z. tau* third instars from wild strains of Palampur (India), Fujian (China), and Baipayl (Bangladesh) and a laboratory strain from Fujian to cold treatments ($\leq$ 1.70 ºC for 3, 8, 10, 13, 15,16, 18, and 20 days) showed that Fujian-wild and Palampur were the most cold-tolerant populations, although the differences at $>$ 99.9% mortality, the level that treatments must achieve, were not significant at the 95% confidence level. Confirmatory tests exposing $>$ 30,000 third instars from Fujian-wild and Palampur to average temperatures ranging from 1.19 to 1.45 ºC for 22 days yielded four survivors, however, none of the four were able to develop to the adult stage. Despite the low levels of infestation observed in *C. sinensis*, *Z. tau* could infest intact harvested oranges placed on trees under field cage conditions and sustain its development to the adult stage. Even though acute larval mortality has been considered the endpoint of cold treatments for tephritids, the low susceptibility and suitability of oranges combined with the cold-treated survivors' inability to develop into adults may be relevant factors for evaluating whether 22 days exposure to $\leq$ 1.70 ºC provides quarantine security for *Z. tau*. This study provides some confidence that future taxonomic divisions that may result within the *Z. tau* complex might be controlled by generic cold treatments that would have the same time duration at a given temperature.

1. Introduction

Phytosanitary treatment of fresh produce constitutes an effective strategy to achieve quarantine security against insect pests. Cold treatment is one of the most applied chemical-free procedures for disinfestation of fruit and vegetables attacked by tephritid and lepidopteran species (Heather and Hallman, 2008). For imports into the USA, cold treatment schedules designated to control citrus pests are as short as 11 days at $\leq$ 0 °C for *Anastrepha* spp., (with the exception of the Mexican fruit fly *A. ludens*), or as long as 22 days at $\leq$ −0.55 °C for the false codling moth *Thaumatotibia leucotreta*, three *Ceratitis* species, and the oriental fruit fly *Bactrocera dorsalis* (USDA, 2022). Although existing cold treatment schedules cover a wide range of tephritid taxa, there are uncertainties about the efficacy of these schedules for putative species within some cryptic complexes.

Comparative studies evaluating the extent to which populations of

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fruit fly complexes respond differently to cold phytosanitary treatments are necessary but challenging, due to quarantine restrictions limiting the movement of live insects between countries (IPPC, 1999). Fortunately, this comparative approach can be applied in the Insect Pest Control Laboratory (IPCL) of the Joint Food and Agriculture Organization of the United Nations/ International Atomic Energy Agency (FAO/IAEA) Centre of Nuclear Techniques in Food and Agriculture as has been done there on previous occasions to determine to what extent phytosanitary treatments could be broadly applied (Hallman et al., 2013; 2019; Myers et al., 2016; Dias et al., 2020).

Zeugodacus tau is a major pest species complex capable of attacking more than 100 host plants (Liquido et al., 2016). Phytosanitary treatments are needed to ship fruit of some of these host plants out of areas where populations of the complex exist. The status of the cryptic species complex of *Z. tau* has been supported by morphological (Zaelor and Kitthawee, 2018), morphometric (Kitthawee and Dujardin, 2010; Kitthawee and Rungsri, 2011), cytological (Baimai et al., 2000), and molecular genetic (Jamnongluk et al., 2003; Thanaphum and Thaenkham, 2003; Zaelor and Kitthawee, 2018; Kitthawee and Julsirikul, 2019) studies. For example, in Thailand, at least nine morphotypes have been identified (Kitthawee and Julsirikul, 2019). The morphotype A from Thailand along with the taxa from Bangladesh, China, India, Japan, Laos, Malaysia, and Sri Lanka have been suggested as *Z. tau* sensu stricto (Yong et al., 2017). As insect populations may differ in thermal tolerance to ecologically relevant temperatures (Sørensen et al., 2001; Ma et al., 2014), assessing whether *Z. tau* populations distributed across a broad geographic range can diverge in their tolerance to cold temperatures used as phytosanitary treatments is essential.

The phytosanitary treatment options available for *Z. tau* comprise the irradiation doses of 72 and 85 Gy (IPPC, 2022a) and two cold treatment schedules, T107-n and T107-o, recently included in the United States Department of Agriculture (USDA) Treatment Manual (USDA, 2022). The schedule T107-n ($\leq$ 1 °C for 17 days) is the only stand-alone cold treatment schedule currently available for *Z. tau* and applies only for guavas. The schedule T107-o (18, 20, and 22 days at $\leq$ 0.56, 1.11, and 1.67 °C, respectively) can only be used as part of a systems approach, which includes registered places of production, trapping requirements, and grove sanitation, to treat citrus from China. The scarce number of approved phytosanitary treatments for *Z. tau* (Jaleel et al., 2018) combined with its high dispersal capacity and invasive potential (Ohno et al., 2008; Shi et al., 2014) pose a serious threat to agricultural production systems. Due to the taxonomic uncertainties around the *Z. tau* complex and the limited cold treatment schedules available, it is of paramount importance to evaluate whether its populations respond similarly to cold treatments. If these populations respond similarly to cold treatments, generic cold treatments that would be efficacious across these populations may be proactively suggested for the entire complex. Generic treatments are particularly important for cryptic species complexes in which new species are likely to be described, as these new species may not be covered by existing phytosanitary treatment schedules.

The objectives of this study were to first determine the most tolerant stage among eggs and larvae and then compare the tolerance of *Z. tau* from wild strains of Baipail (Bangladesh), Fujian (China), and Palampur (India) and a laboratory strain from Fujian, to a cold phytosanitary treatment ($\leq$ 1.70 °C) carried out with infested oranges. The second objective was to evaluate the schedule T107-o as a stand-alone cold treatment for *Z. tau* in citrus in confirmatory tests using infested navel oranges with $>$ 30,000 third instars of the most tolerant population identified in the exploratory tests. Additionally, we conducted field cage trials to evaluate the susceptibility and suitability of oranges to *Z. tau* in semi-natural conditions using populations from Bangladesh, China, and India. *Citrus sinensis* has been reported as a host of *Z. tau* based on trade and interception data (CASI, 1994; Wu et al., 2009; GAQSIQ, 2011; Huang, 2017). However, host preference and suitability tests have shown that citrus is less susceptible to *Z. tau* than cucurbit fruit under laboratory and field conditions (Lin et al., 2005; Wu et al., 2011).

2. Material and methods

2.1. Origin and maintenance of the insects

Four populations of the *Z. tau* complex from Bangladesh, China, and India were evaluated in our study (Table 1). Tests were conducted using the same colonies over at least nine generations without the addition of wild insects. Fruit fly colonies were maintained at the IPCL of the Joint FAO/ IAEA Centre of Nuclear Techniques in Food and Agriculture in Seibersdorf, Austria. Voucher specimens from the four *Z. tau* populations were periodically deposited at the IPCL. Immature stages were reared on zucchinis (*Cucurbita pepo*) infested for up to 2 h by sexually mature *Z. tau* females. Infested zucchinis were transferred to plastic trays (46.0 $\times$ 35.0 $\times$ 17.0 cm) filled with sawdust (GOLDSPAN® smoke, Germany) to allow for larval development (5–6 days). After 10–15 days, *Z. tau* puparia from each population were collected from the plastic trays and placed into different screen-mesh cages (45.0 $\times$ 45.0 $\times$ 45.0 cm) for adult emergence. Adults were maintained in screen-mesh cages with access to water and a dry artificial diet (1 hydrolysed yeast: 3 sucrose) (Vargas et al., 1984). All insects were reared under laboratory conditions at 23 $\pm$ 1 °C, 65 $\pm$ 5% RH, and 12 L:12D photoperiod.

2.2. Insect identification

Representative male and female adults of *Z. tau* from Bangladesh, China, and India were preserved in 96% ethanol and independently identified by experts. Samples sent to Marc De Meyer (Royal Museum for Central Africa, Tervuren, Belgium) were morphologically identified as *Z. tau* sensu stricto. De Meyer's morphological identification was further confirmed by Kenny Meganck (Royal Museum for Central Africa) through mitochondrial DNA barcoding using the CO1 region. DNA barcoding was independently performed by Norman Barr (USDA, Animal and Plant Health Inspection Service – APHIS, Texas, USA) and confirmed as belonging to morphotype A within the *Z. tau* complex. The CO1 barcode sequences used by Barr to confirm the morphotype of the *Z. tau* populations used in our study were deposited at GenBank with accession codes ON081959-ON081962 and ON113500-ON113502.

2.3. Host susceptibility and suitability tests

The potential for wild populations of *Z. tau* from Baipayl, Fujian, and Palampur to infest *C. sinensis* was evaluated in no-choice tests using field cages (2.0 $\times$ 3.0 m) containing a potted *C. senensis* plant under semi-natural conditions. Before the experiment and within 48 h after adult emergence, 100 couples from each *Z. tau* population were separated and maintained in three acrylic cages (29.0 $\times$ 20.0 cm) with access to water and a dry artificial diet (3-parts sugar: 1-part yeast hydrolysate). The experiments were conducted when the insects reached the peak of sexual maturity, approximately 30 days after emergence. One day before the experiment, four units of detached zucchini, intact oranges, or punctured oranges, three water sources and three diet feeders were individually placed in holders made of galvanized steel mesh containing elastic straps and hung on the potted plant branches. Oranges were

Table 1
Collection site and host fruit collected for the four populations of the *Zeugodacus tau* used in cold treatment experiments conducted across multiple generations.

Population (country)	Latitude	Longitude	Host	Generations
Baipayl (Bangladesh)	23°57'35"	90°16'54"	*Lagenaria siceraria*	F-06 to F-16
Fujian-laboratory (China)	26°05'06"	119°14'23"	*Solanum melongena*	F-25 to F-34
Fujian-wild (China)	26°05'06"	119°14'23"	*Luffa aegyptiaca*	F-03 to F-16
Palampur (India)	30°54'30"	77°05'59"	*Cucumis sativus*	F-03 to F-13

punctured in four different places around the pedicel scar using four stainless steel dissecting needles to mimic oviposition wounds in an infested fruit. The next morning at 9:00, 100 couples of *Z. tau* from the same origin were released into the field cages containing oviposition (zucchini, intact orange, or punctured oranges) and food (water and diet) resources. Insect mortality was monitored and recorded during the experiment, and dead flies were replaced with individuals from the same cohort kept under the same conditions. All fruit were removed from the field cages on the next day at 9:00, weighed, and transferred to individually labelled plastic containers (9.5 × 9.5 × 11.5 cm for oranges and 21.0 × 21.0 × 14.5 cm for zucchinis).

When larvae were seen leaving the commodities, zucchinis and oranges were transferred to labelled plastic trays (43.5 × 28.0 × 17 cm) containing ~320 g of sawdust mixed with 50 ml water. The oranges and zucchinis remained untouched for 20 and 12 days after the experiment, respectively, as these were the developmental periods required for the pupariation of most larvae. After the larval development period, all insects found outside each commodity were recorded and transferred to labelled plastic containers (9.5 × 9.5 × 11.5 cm) with 15 g of moist sawdust. All zucchinis and oranges were dissected approximately three and four weeks after their infestation, respectively. During the fruit dissection, the numbers of dead larvae, live larvae, and puparia found inside the commodities were recorded. Live insects found as larvae or puparia were carefully transferred to plastic containers with sawdust previously labeled according to their treatment. After adult emergence, insects were sexed and counted. The numbers of fully formed adults, deformed adults, partially emerged adults, and dead puparia were recorded for each treatment. Furthermore, the percentage of infested hosts, the number of larvae per host, and the number of non-deformed adults that emerged from all insects recorded (live larvae, dead larvae, and puparia) were calculated and compared among the *Z. tau* populations. Non-infested zucchinis, intact oranges, and punctured oranges were included in the calculations by considering the number of larvae or emerged adults per fruit equal to zero.

2.4. Most tolerant stage studies

Fruit of zucchini are a preferred host of *Z. tau* (Lin et al., 2005; Wu et al., 2011; Khan et al., 2011) and were used to determine most tolerant stage to cold treatment. Zucchini grown locally in the Vienna area were washed, rinsed, soaked for 15 min in antifungal solution (4% sodium benzoate, 1% sodium hypochlorite), re-rinsed and dried. They were then exposed for 2 h to ~1000 sexually mature *Z. tau* adults from Fujian, China, held in screen-mesh cages (45 × 45 × 45 cm).

After 4 days *Z. tau* reared on zucchini began to pupariate. Therefore, zucchini infested right after removal from the infestation cage and 1, 2, 3, and 4 days later were separately placed at 1 ± 0.1 °C for 7 days. Cold treatment of infested zucchini was conducted in a 2 m^3 environmental chamber (model SE-2000–4, Thermotron Industries, Holland, MI) as described by Hallman et al. (2013). Cold-treated zucchini fruit infested with different life stages were held at 25 ± 1 °C for 7 days post-infestation and then examined for evidence of insect development. Therefore, those treated as eggs would have had to hatch and continue developing to observable size (third and possibly late second instar) to be considered survivors. For each experimental unit there were five control fruits and five treated ones. The number of replicates varied between five and eight (Table 2).

2.5. Orange infestation for cold treatment

Navel oranges, *C. sinensis*, imported from Israel, Spain, and South Africa were acclimated to room temperature and sanitized before and after infestation to prevent fungal contamination. Before infestation, oranges were washed, rinsed, soaked for 15 min in antifungal solution (4% sodium benzoate, 1% sodium hypochlorite), re-rinsed and dried. Sanitized oranges were punctured on the pedicel side with four needles (1.0 mm diameter) to stimulate oviposition by *Z. tau* females. Natural infestation consisted of exposing four to six punctured oranges for 6 h to ~2000 sexually mature *Z. tau* adults held in screen-mesh cages (45.0 × 45.0 × 45.0 cm). Females from all *Z. tau* populations used in our study reached the peak of sexual maturity approximately 30 days after adult emergence. Infested oranges were re-sanitized with antifungal solution, rinsed, and dried for immediate biometric screening. After the re-sanitization of infested oranges, each fruit was weighted using a digital balance (model IS 32001, VRW, Italy) and its perimeter measured. Infested oranges ($\bar{x}_{weight}$ = 265.74 ± 0.83 g, $\bar{x}_{perimeter}$ = 25.83 ± 0.03 cm [mean ± SE]) were individually placed into plastic containers (9.5 × 9.5 × 11.5 cm) and incubated in a large capacity reach-in environmental chamber (model 3951, ThermoFisher, United States) set at 25 °C for 7–8 days until the larvae reached the third instar. Larval development time from egg to mid-third instar in oranges kept at 25 °C was four days longer than in zucchinis.

2.6. Cold treatment of infested oranges

Approximately one hour before initiating the cold treatment, infested oranges were inspected and any wandering larvae or puparia found outside the fruit were removed. Oranges were placed in individually labelled plastic containers (9.5 × 9.5 × 11.5 cm) with lids that included an opening covered by voile fabric and grouped in plastic trays according to the treatment duration. The containers with oranges infested by different populations were randomized within each plastic tray. The plastic trays with the infested oranges were then placed in a 2 m^3 environmental chamber (as described above). The position of the plastic trays was randomized within the environmental chamber for every replication. Airflow within the chamber was approximately 28.3 m^3/min. The chamber temperature was set to 0.6 °C to achieve the target fruit pulp temperatures of 1.70 °C or below (hereafter referred to as ≤ 1.70 °C). The treatment temperature was chosen to align with the current USDA treatment schedule T107-o for the control of *Z. tau* and other *Bactrocera* and *Zeugodacus* spp. associated with consignments of *C. sinensis* (USDA, 2022). Exploratory testing conducted with cold exposure periods of 3, 8, 10, 13, 15, 16, 18, and 20 days was used to determine the treatment duration required to achieve 100% mortality of third instars from all four *Z. tau* populations. Comparison of LT estimates for probit 9 mortality at 95% CI were used to select the most cold-tolerant *Z. tau* populations to be used in the confirmatory tests. A confirmatory test was then done using 41,331 *Z. tau* third instars from China and India in infested oranges at ≤ 1.70 °C for 22 days. Experimental procedures used in our study followed the recommendations of

Table 2
Percentage of *Zeugodacus tau* third instars recovered from zucchinis treated at 1 ± 0.1 °C for 7 days. Mortalities in control replicates were 1.1%, 1.0%, 8.1%, and 0.3%.

Age (days old)	Survival (%)								
	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Rep. 5	Rep. 6	Rep. 7	Rep. 8	Mean ± SE
0 (young egg)	0	0	1.40	0	1.37	0	-	-	0.46 ± 0.29
1	0	0	0	0	0	8.48	-	-	1.41 ± 1.41
2	0	0	0.87	3.34	0	-	-	-	0.84 ± 0.65
3	0	2.00	0	0	0.59	-	-	-	0.52 ± 0.39
4 (3rd instar)	7.63	4.00	0	0	0	3.70	0.25	6.62	2.78 ± 1.12

the Phytosanitary Measures Research Group (PMRG) guideline for cold treatments (PMRG, 2019). The efficacy level of the cold treatment evaluated in our confirmatory test was calculated with a 95% confidence according to Couey and Chew (1986). The total number of insects treated considered in the efficacy calculation was adjusted based on the control mortality for each replication.

2.6.1. Temperature measurement

Temperatures inside the environmental chamber were recorded every 15 min using two four-channel data loggers (HOBO® UX120–06 M, Onset Computer Inc., USA) with type-T thermocouples (TMCx-HD, Onset Computer Inc., USA) each. Thermocouples were periodically calibrated using an ice-water solution as reference temperature, immediately before the start of the tests (ASTM E563–11). Calibration readings were recorded four times at 5-minute intervals for each thermocouple. Mean calibration factors were 0.04 ± 0.02 °C and 0.05 ± 0.02 °C (grand mean ± SEM) for thermocouples used in non-infested fruit and air, respectively. Fruit core temperature was recorded during the treatments using four thermocouples inserted into the center of non-infested oranges. The non-infested oranges were placed in individual plastic containers (9.5 × 9.5 × 11.5 cm) covered with lids containing a central hole to allow for the insertion of the temperature probe into the fruit core. Water and air temperatures were recorded using one and three thermocouples, respectively, positioned in different points of the environmental chamber. The treatment started when at least two temperature probes inserted into the non-infested oranges reached ≤ 1.70 °C. This was also used to identify the end of the cool-down period.

2.6.2. Fruit dissection and insect evaluation

Following treatment, infested oranges were held at 25 ± 1 °C for at least 24 h before dissection to provide time for larval recovery. Untreated controls were also held at 25 ± 1 °C for at least 24 h from the time the infested oranges were placed into the cold chamber. Moving larvae found during fruit dissection were counted as survivors and immediately transferred to individually labelled plastic containers (6.5 × 6.5 × 8 cm) filled with a thin layer of moist sawdust. Non-moving third instars were considered dead, but any larvae that were of normal colouration were held in plastic containers (6.5 × 6.5 × 4 cm) containing moist sawdust for further evaluation. Dead puparia, partially emerged adults, deformed and fully formed adults were counted ≥ 6 weeks after fruit dissection, when non-moving larvae with normal colouration were re-checked to confirm their death. A minimum of seven replicates were conducted for each treatment duration in the exploratory tests. In confirmatory tests, a total of 41,331 third instar larvae in infested oranges were treated for 22 days.

2.7. Statistical analysis

In the host susceptibility and suitability tests, the percentage of infested fruit, number of larvae per fruit, and number of emerged adults per fruit were evaluated using Poisson generalized linear mixed model (GLMM) with fruit, population, and their interaction (×) as fixed effects and replicate as a random effect. In the exploratory tests, larval survival was adjusted using Abbott's correction for control mortality and analysed using a binomial GLMM. The duration of the cold treatment (dose), population, and their interaction (×) were modelled as fixed effects. Because an incomplete block design was used in our study, block and replication were modelled as random effects. The statistical significance of the fixed effects and their interaction were calculated based on Wald chi-square test with type III sums of squares. Post hoc pairwise comparisons of estimated marginal means between dose × population were performed with Bonferroni adjustment (Holm, 1979). Model selection was performed using Akaike's information criterion (Burnham and Anderson, 2002). Aiming to compare cold tolerance among *Z. tau* populations, a probit model with adjustment for overdispersion was used to estimate the lethal time (LT) of cold exposure to achieve 99.9% and 99.9968% (probit 9) mortality and their fiducial limits at 95% CI, as described by Dias et al. (2020). Data from unexposed insects were included in the probit model. The LT values were then compared among populations using lethal dose ratio tests (Robertson et al., 2016). All statistical analyses were performed in R (version 4.0.5; R Core Team, 2021) and R Studio (version 1.4.1106; RStudio Team, 2021). The GLMM, post hoc, and model selection analyses were carried out using *lme4* (Bates et al., 2015), *emmeans* (Lenth, 2022), and *bbmle* (Bolker and R Development Core Team, 2021), respectively. Probit and lethal dose ratio tests were conducted using the *drc* (Ritz et al., 2015) and *multcomp* (Hothorn et al., 2008) packages.

3. Results

3.1. Host susceptibility and suitability tests

The percentages of fruit infested by each *Z. tau* population are shown in Fig. 1A. Regardless of origin, *Z. tau* females showed a higher propensity to infest zucchinis, followed by punctured oranges and intact oranges, the latter the least infested host (host: $\chi^2 = 70.90$; df = 2; $P < 0.0001$). Females from Baipayl infested intact oranges more often than females from other populations and, together with Fujian-wild females, exhibited a higher propensity to infest punctured oranges compared with Palampur females (population: $\chi^2 = 23.22$; df = 2; $P < 0.0001$ and population × host: $\chi^2 = 40.62$; df = 4; $P < 0.0001$).

The numbers of *Z. tau* larvae found in zucchinis, intact oranges, and punctured oranges are shown in Fig. 1B. Hosts infested by females from Baipayl or Fujian-wild yielded higher numbers of larvae as compared with fruit infested by Palampur females (population: $\chi^2 = 62.03$; df = 2; $P < 0.0001$). Infested zucchinis yielded the highest numbers of larvae, followed by punctured oranges and intact oranges (host: $\chi^2 = 1710.79$; df = 2; $P < 0.0001$). Zucchinis infested by Fujian-wild females yielded the highest number of larvae among all hosts and populations, but larvae infesting either intact or punctured oranges were found in higher numbers for Baipayl compared with Fujian-wild and Palampur (population × host: $\chi^2 = 1403.72$; df = 4; $P < 0.0001$).

Immature stages of *Z. tau* populations infesting zucchinis yielded the highest numbers of fully formed adults, followed by punctured oranges and intact oranges (host: $\chi^2 = 1494.45$; df = 2; $P < 0.0001$). Zucchinis infested by the *Z. tau* population from Fujian-wild exhibited the highest average number of emerged adults per fruit, followed by Baipayl and Palampur. Intact and punctured oranges infested by *Z. tau* from Baipayl showed higher numbers of emerged adults than Fujian-wild and Palampur (population: $\chi^2 = 40.59$; df = 2; $P < 0.0001$ and population × host: $\chi^2 = 1011.10$; df = 4; $P < 0.0001$).

3.2. Most tolerant stage

After seven days at 1 ± 0.1 °C, survival of *Z. tau* immatures in zucchini fruit was nominally highest at 4 days, which would be mostly third instars (Table 2). However, the probability value in analysis of variance for all ages was 0.37, regardless of whether the data were analyzed as percentage or after arc-sin transformation. Nevertheless, concluding that there are no differences among stages in phytosanitary treatment research when there actually are, may lead to the development of a treatment that will be less efficacious than required, while concluding there are differences when there are not, does not carry risk of a sub-efficacious treatment resulting, if the stage with the nominally highest rate of survival is used to develop the treatment. Therefore, for phytosanitary treatment research it is better to do the research on the stage that gave the highest mean survival, even if it is not significant, and that is definitely the third instar in this case.

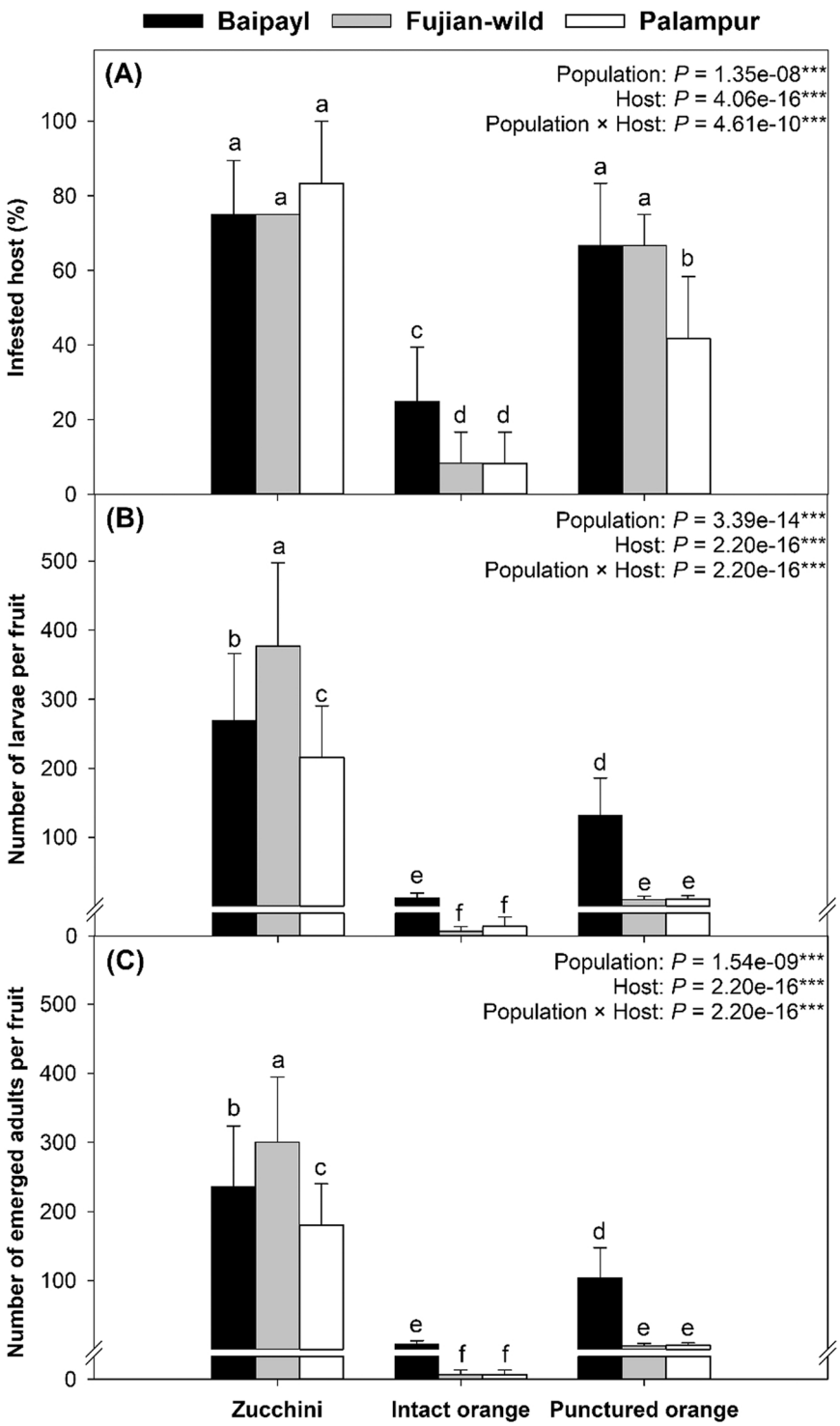

Fig. 1. Levels of attractiveness and suitability of zucchini, intact orange, and punctured oranges for *Zeugodacus tau* populations from Baipayl (Bangladesh), Fijian-wild (China), and Palampur (India). (A) Percentage of infested hosts (mean ± SE) in all replications (Poisson GLLM). (B) Number of third instars (mean ± SE) yielded per host fruit (Poisson GLMM). (C) Percentage of fully formed adult per fruit emerged (mean ± SE) from insects reared on different hosts (LMM). Bars followed by different letters indicate statistically significant differences (least-squares means, $P < 0.05$).

3.3. Exploratory testing

Treatment temperatures recorded for fruit core, air, and water are summarized in Tables S1 and S2. The fruit core temperature recorded during the treatment was 1.40 ± 0.02 °C (mean ± SEM). The time for cooling down the non-infested oranges to ≤ 1.70 °C was 533.25 ± 32 min (mean ± SE). Corrected mortality of *Z. tau* third instars from Baipail, Fujian-wild, Fujian-laboratory, and Palampur exposed to cold treatment for 3–20 d is shown in Table 3. Differential population responses to cold exposure were observed in *Z. tau* third instars treated for up to 15 d at ≤ 1.70 °C. Fujian-wild was the most cold-tolerant population in treatments of 13 and 15 d, and Palampur was more cold-tolerant than populations from Baipayl and Fujian-laboratory in treatments of 3 and 8 d (dose × population: $\chi^2 = 570.02$; df = 24; $P < 0.0001$). *Zeugodacus tau* from Baipayl was the most susceptible population to cold treatment (population: $\chi^2 = 74.24$; df = 3; $P < 0.0001$). There was no statistically detectable difference in mortality among *Z. tau* populations exposed to ≤ 1.70 °C for 16, 18, and 20 d, but a few third instars survived after exposure to those treatments; however, none were able to develop to the adult stage (Table 3, Table 4). In fact, *Z. tau* third instars were only able to complete their development and emerge as adults when exposed to cold treatment for up to 13 days,

Table 3
Numbers of replicates, treated fruit, treated larvae, live larvae, larvae per fruit, and corrected mortality (%) of four populations of the *Zeugodacus tau* complex third instars reared in oranges exposed to 1.40 °C ± 0.02 (mean ± SEM) for 0–20 days.

Treatment duration	Population	N	Treated fruit	Treated larvae (adjusted counts)[a]	Live larvae	Larvae/fruit (mean ± SE)	Corrected mortality (mean ± SE)[b]
0 (control)	Baipayl	20	56	3625	3448	65 ± 9	4.28 ± 1.12 A
	Fujian-laboratory	20	76	5618	5280	74 ± 10	7.93 ± 1.31 B
	Fujian-wild	20	95	8418	7605	88 ± 11	7.10 ± 1.33 C
	Palampur	17	70	4862	4567	69 ± 9	5.55 ± 1.03 AC
3 days	Baipayl	10	27	2274 (2206)	1294	84 ± 13	33.06 ± 5.14 A
	Fujian-laboratory	10	25	1306 (1207)	545	52 ± 13	32.16 ± 5.90 A
	Fujian-wild	10	23	2610 (2513)	1249	114 ± 32	27.86 ± 5.98 B
	Palampur	11	25	2117 (1912)	1310	85 ± 18	25.79 ± 4.51 C
8 days	Baipayl	10	24	2330 (2262)	97	97 ± 20	92.87 ± 2.42 A
	Fujian-laboratory	10	25	2090 (1957)	123	84 ± 27	82.02 ± 5.72 B
	Fujian-wild	10	26	1622 (1541)	163	62 ± 11	82.99 ± 4.34 B
	Palampur	10	28	2300 (2091)	314	82 ± 21	78.52 ± 4.43 C
10 days	Baipayl	13	46	3529 (3419)	64	77 ± 10	93.49 ± 3.11 A
	Fujian-laboratory	14	50	3772 (3597)	101	75 ± 16	91.60 ± 2.94 B
	Fujian-wild	15	53	3346 (3084)	85	63 ± 11	96.64 ± 1.04 C
	Palampur	10	33	2708 (2474)	146	82 ± 24	93.08 ± 1.47 B
13 days	Baipayl	9	33	3261 (3134)	27	99 ± 23	98.91 ± 0.38 A
	Fujian-laboratory	9	57	4328 (4034)	50	76 ± 13	98.13 ± 0.58 B
	Fujian-wild	9	59	4908 (4908)	60	83 ± 13	96.22 ± 1.37 C
	Palampur	10	27	2024 (1872)	33	75 ± 15	98.32 ± 0.75 B
15 days	Baipayl	9	36	3371 (3220)	15	94 ± 21	99.19 ± 0.39 A
	Fujian-laboratory	9	36	2629 (2477)	7	73 ± 11	99.88 ± 0.07 B
	Fujian-wild	9	37	3138 (3039)	24	85 ± 13	98.01 ± 1.35 C
	Palampur	10	29	2511 (2314)	4	86 ± 22	99.69 ± 0.21 AB
16 days	Baipayl	9	37	3385 (3247)	1	91 ± 14	99.95 ± 0.05 A
	Fujian-laboratory	10	40	2559 (2401)	0	64 ± 10	100.00 ± 0.00 A
	Fujian-wild	9	35	3557 (3432)	2	102 ± 12	99.93 ± 0.06 A
	Palampur	8	25	2179 (1988)	5	87 ± 18	99.75 ± 0.17 A
18 days	Baipayl	8	37	2122 (1918)	0	57 ± 9	100.00 ± 0.00 A
	Fujian-laboratory	8	41	2574 (2251)	0	63 ± 10	100.00 ± 0.00 A
	Fujian-wild	8	42	2693 (2516)	1	64 ± 12	99.99 ± 0.01 A
	Palampur	7	37	2218 (2090)	2	60 ± 11	99.97 ± 0.02 A
20 days	Baipayl	8	35	1961 (1818)	0	56 ± 9	100.00 ± 0.00 A
	Fujian-laboratory	8	36	2725 (2434)	0	76 ± 12	100.00 ± 0.00 A
	Fujian-wild	8	36	3795 (3615)	1	105 ± 17	99.94 ± 0.06 A
	Palampur	8	36	2904 (2778)	1	81 ± 15	99.99 ± 0.01 A

[a] Adjusted number of treated larvae based on the natural mortality rate of the control group of each replication.
[b] Different letters indicate statistically significant differences between groups (estimated marginal means contrasts from the binomial GLMM, $P < 0.05$)

as observed for treated insects from Fujian-wild and Palampur (Table 4).

Lethal time (LT) estimates for 99.9% and 99.9968% (probit 9) differed significantly among *Z. tau* populations exposed to cold treatments (Table 5). The estimated $LT_{99.9}$ value for Baipayl was the lowest among all populations, followed by Fujian-laboratory, Fujian-wild, and Palampur. At the Probit 9 level of control, Palampur, Fujian-wild, and Fujian-laboratory were equally the most cold-tolerant populations, whereas Baipayl was the most cold-susceptible population (Table 5).

3.4. Confirmatory testing

Both Palampur and Fujian-wild were included in confirmatory tests, but an emphasis was placed on the Fujian-wild population because they were found to be more cold-tolerant than Palampur in the treatment of 22 d at ≤ 1.70 °C (dose × population: $\chi^2 = 8604.9$; df = 1; $P < 0.0001$). Additionally, the control mortality was lower for oranges infested with 9014 third instars from Fujian-wild (~10% mortality) than 1321 larvae from Palampur (~21% mortality) in our confirmatory tests (population: $\chi^2 = 147{,}233.4$; df = 1; $P < 0.0001$, Table 6).

A total of 36,512 third instars from Fujian-wild and 4819 third instars from Palampur were exposed to ≤ 1.70 °C for 22 days (Table 6). Four apparent survivors from the Fujian-wild population were observed during the post-treatment evaluation. Of those four survivors, two were found as puparia inside the containers with the treated oranges, one as a moving larva inside the commodity, and one as a live colour larva which pupariated during the holding period. None of the four survivors developed to the adult stage. The treated insect numbers from Fujian-wild and Palampur were adjusted based on the mortality in the control groups, summed, and calculated as 35,275 with the resulting efficacy of 99.9915% at the 95% confidence level.

4. Discussion

The confirmatory tests in this study demonstrated, with a high level of confidence, that the cold treatment of 22 days at ≤ 1.70 °C prevented *Z. tau* from developing to the adult stage. This result supports the application of this treatment for mitigating the risk of introduction and establishment of *Z. tau* through the imported citrus pathway. The 22-day cold treatment did not, however, prevent four larvae from developing to the puparial stage. This may pose a challenge for quarantine inspection

Table 4
Number of insects recovered from navel oranges treated at 1.40 ± 0.02 °C (mean ± SEM) for 0–20 days.

Treatment duration	Population	Dead larvae		Live larvae		Puparia		Insects emerged as adults
		Live colour	Dark colour	Crawling	Slow moving	Non-deformed	Deformed	
0 (control)	Baipayl	161	16	3300	81	65	2	1844
	Fujian-laboratory	294	44	5111	97	67	5	3084
	Fujian-wild	610	203	7261	269	71	4	3856
	Palampur	244	51	4423	87	19	38	2381
3 days	Baipayl	608	372	1102	109	57	26	387
	Fujian-laboratory	405	356	436	42	50	17	191
	Fujian-wild	846	515	1066	101	59	23	414
	Palampur	476	331	1076	205	25	4	575
8 days	Baipayl	504	1729	36	53	4	4	0
	Fujian-laboratory	500	1467	52	58	4	9	8
	Fujian-wild	547	912	42	111	9	1	12
	Palampur	1045	941	163	144	3	4	27
10 days	Baipayl	705	2760	9	49	6	0	75
	Fujian-laboratory	765	2906	48	43	10	0	4
	Fujian-wild	536	2725	33	40	12	0	2
	Palampur	581	1981	52	91	3	0	1
13 days	Baipayl	321	2913	2	4	19	2	0
	Fujian-laboratory	873	3405	6	34	10	0	0
	Fujian-wild	1043	3805	6	39	14	1	1
	Palampur	568	1423	1	26	6	0	1
15 days	Baipayl	403	2953	0	4	4	7	0
	Fujian-laboratory	494	2128	0	1	5	1	0
	Fujian-wild	790	2324	11	4	5	4	0
	Palampur	474	2033	1	2	1	0	0
16 days	Baipayl	269	3115	0	1	0	0	0
	Fujian-laboratory	364	2195	0	0	0	0	0
	Fujian-wild	392	3163	0	2	0	0	0
	Palampur	363	1811	1	1	0	3	0
18 days	Baipayl	151	1971	0	0	0	0	0
	Fujian-laboratory	241	2333	0	0	0	0	0
	Fujian-wild	289	2403	0	0	1	0	0
	Palampur	298	1918	1	1	0	0	0
20 days	Baipayl	182	1779	0	0	0	0	0
	Fujian-laboratory	231	2494	0	0	0	0	0
	Fujian-wild	324	3470	0	0	1	0	0
	Palampur	225	2678	1	0	0	0	0

Table 5
Probit model estimates and 95% fiducial limits of days cold treatment at 1.40 ± 0.02 °C (mean ± SEM) required to produce 99.9% and 99.9968% mortality of *Zeugodacus tau* complex third instars in oranges.

Population	Slope ± SE[a]	LT[b] (95% fiducial limits) in days	
		$LT_{99.9}$	$LT_{99.9968}$ (probit 9)
Baipayl	1.86 ± 0.08	17.22 (16.46, 17.98) A	28.02 (26.37, 29.67) A
Fujian-laboratory	1.50 ± 0.09	20.93 (19.80, 22.07) B	38.37 (35.41, 41.34) B
Fujian-wild	1.56 ± 0.06	21.54 (20.57, 22.50) BC	38.58 (36.21, 40.94) B
Palampur	1.70 ± 0.07	23.03 (22.02, 24.05) C	39.32 (36.99, 41.64) B

[a] Log-normal model with lower and upper limits at 0 and 1, respectively. Heterogeneity = 8.15.
[b] Lethal time (LTs followed by different letters indicate statistical significance, lethal dose ratio tests, $P < 0.05$)

as cold treatments are typically proposed based on efficacy data targeting acute larval mortality as the treatment endpoint (PMRG, 2019). Thus, any live life stages of regulated fruit fly species found post-treatment would be considered a treatment failure (IPPC, 2015; IPPC, 2017; IPPC, 2022b). The presence of live larvae is, however, regularly accepted in many phytosanitary irradiation treatments that do not rely on larval mortality as the endpoint for treatment efficacy. Similarly, cold treatments for several citrus species against *C. capitata* have also been accepted as international standards (i.e., PTs 24–26 from ISPM 28) based on research that considered prevention of pupariation as the end-point De Lima et al., 2007).

In the present case, the 22-day cold treatment for *Z. tau* is equivalent to the most severe cold treatment in use for *A. ludens* in citrus (USDA, 2022). Extending the treatment duration or lowering the treatment temperature would risk damage to the citrus in-transit, which would be an impediment to commercial adoption. Another important consideration is that the current use of the USDA treatment schedule T107-o includes additional measures to reduce the likelihood that *Z. tau* would be in the pathway, further reducing the overall risk (USDA,

Table 6
Confirmatory test with *Zeugodacus tau* third instars from Fujian-Wild and Palampur treated at 1.40 ± 0.02 °C (mean ± SEM) for 22 days in oranges.

Treatment duration	Population	N	Treated fruit	Treated larvae (adjusted counts)[a]	Live larvae	Larvae/fruit (mean ± SE)	Mortality (mean ± SE)[b]
0 (control)	Fujian-Wild	7	65	9014	8037	139 ± 19	10.23 ± 1.54 A
	Palampur	4	13	1321	1074	102 ± 29	21.18 ± 8.19 B
22 days	Fujian-wild	7	242	36,512 (31,433)	4	151 ± 9	99.99 ± 0.00 A
	Palampur	4	68	4819 (3842)	0	71 ± 11	100.00 ± 0.00 B

[a] Adjusted number of treated larvae based on the natural mortality rate of the control group of each replication.
[b] Different letters indicate statistically significant differences between groups (estimated marginal means contrasts from the binomial GLMM, $P < 0.05$)

2022).

Finally, host suitability is an important consideration when evaluating the risk of introduction of *Z. tau* to pest-free areas through orange consignments. The *Z. tau* populations tested in our study showed poor infestation levels and low adult emergence in orange compared with zucchini. Of the three populations evaluated in the host suitability experiments, Baipayl performed slightly better on intact orange compared to the other populations. Conversely, Baipayl was less tolerant to cold treatments over the middle range of treatment durations (e.g. 3–15 day treatment durations) as compared with the other groups. This suggests that while Baipayl may be more likely to occur in the commodity, it is also most susceptible to the treatment. Of the two populations chosen for the confirmatory tests, based on their tolerance relative to the other populations Fujian-wild was significantly less likely to infest intact orange and Palumpur was significantly less likely to infest punctured fruit. The number of larvae per fruit in both Fujian-wild and Palumpur was significantly less than for Baipayl. Additionally, the use of detached oranges in these trials was a matter of practicality and does not suggest attached fruit would be similarly utilized in commercial citrus groves. At the time of writing, we could not find documented observations of *Z. tau* infesting fruit in citrus production areas in the scientific literature (Lin et al., 2005; Li et al., 2020). This suggests that infestations in the field are likely rare, and, subsequently, the occurrence of infested fruit in the pathway is further diminished due to low host preference and performance in citrus.

5. Conclusions

Our results demonstrate that the exposure of *Z. tau* third instars infesting *C. sinensis* to $\leq 1.70\ ^{\circ}C$ for 22 days will prevent *Z. tau* from developing to the adult stage with a high level of confidence. Four third instars out of $> 40{,}000$ cold treated individuals were able to pupariate but failed to emerge as adults. Including the cold treatment of $\leq 1.70\ ^{\circ}C$ for 22 days as part of a systems approach would provide an additional safety measure and a more conservative option, considering that treated insects are unable to emerge as viable adults and infestation of harvested orange by *Z. tau* is low. It remains to be determined the extent to which differential responses of *Z. tau* populations to cold treatments found in our study correlate with phylogenetic differences. However, this study provides confidence for plant protection organizations that the use of a single cold treatment will provide quarantine level control regardless of future taxonomic divisions that may occur within the *Z. tau* complex.

CRediT authorship contribution statement

Vanessa S. Dias: Conceptualization, Methodology, Formal analysis, Investigation, Visualization, Data curation, Writing – original draft, Writing – review & editing, Supervision. **Guy J. Hallman:** Conceptualization, Methodology, Investigation, Data curation, Writing – original draft, Writing – review & editing, Supervision. **Alexandre S. Araújo:** Methodology, Investigation, Data curation. **Inajara V.G. Lima:** Investigation, Data curation. **Fabio L. Galvão-Silva:** Investigation, Data curation. **Luis A. Caravantes:** Investigation, Data curation. **Martha N. G. Rivera:** Investigation, Data curation. **Jhonatan S. Aguilar:** Investigation, Data curation (host susceptibility and suitability tests). **Carlos E. Cáceres-Barrios:** Conceptualization, Project administration, Writing – review & editing. **Marc J.B. Vreysen:** Conceptualization, Project administration, Writing – review & editing. **Scott W. Myers:** Conceptualization, Methodology, Project administration, Writing – original draft, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.postharvbio.2023.112392.

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